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REVIEW

Emerging roles of the SUMO pathway in development

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Abstract Sumoylation is a reversible post-translational modification that targets a variety of proteins mainly within the nucleus, but also in the plasma membrane and cytoplasm of the cell. It controls diverse cellular mechanisms such as subcellular localization, protein–protein interactions, or transcription factor activity. In recent years, the use of several developmental model systems has unraveled many critical functions for the sumoylation system in the early life of diverse species. In particular, detailed analyses of mutant organisms in both the components of the SUMO pathway and their targets have established the importance of the SUMO system in early developmental processes, such as cell division, cell lineage commitment, specification, and/or differentiation. In addition, an increasing number of developmental proteins, including transcription factors and epigenetic regulators, have been identified as sumoylation substrates. Sumoylation acts on these targets through various mechanisms. For example, this modification has been involved in converting a transcription factor from an activator to a repressor or in regulating the localization and/or stability of numerous transcription factors. This review will summarize current information on the function of sumoylation in embryonic development in different species from yeast to mammals.

Introduction

Covalent conjugation of SUMO (sumoylation) is a reversible modification that can change and regulate the function of a protein. It involves the attachment of a short polypeptide called SUMO (small ubiquitin-related modifier). This modifier is related in two aspects to ubiquitin: it resembles the three-dimensional structure of ubiquitin [1–3], and they both share a common mechanism to attach to proteins [4–6]. However, different functional outcomes derive from these two types of modification. Ubiquitin modification is mainly associated with proteasome-mediated degradation of proteins, although this regulated degradation also plays a critical role in regulating biological processes [7–9]. On the other hand, SUMO modification can impact protein activity, localization, and stability. Sumoylation has emerged as a key regulatory cellular mechanism that is involved in a variety of processes including cell cycle regulation, transcription, nuclear architecture, chromosome stability, and subcellular transport [10–13].

SUMO polypeptides are conserved throughout the eukaryotic kingdom. Although single genes encoding SUMO have been described in yeast and other invertebrates, at least three SUMO genes are known in mammals. SUMO1, 2, and 3 are ubiquitous proteins found in all developmental stages [10, 11]. SUMO1 does not form poly-SUMO chains, but SUMO2 and 3 do [14, 15]. A fourth gene encoding SUMO4 in the human genome is predominantly expressed in immune tissues [16].

Sumoylation is a reversible reaction. Specific proteases that act in vivo to remove SUMO from substrates have been described [11, 17]. In most cases, the modification involves only a small proportion of the total pool of a target protein, which is enough to produce a significant effect [18]. The attachment of SUMO to proteins is highly unstable, and sumoylated proteins are often present at a level below normal detection limits. Therefore, sumoylation remained unknown for many years after other posttranslational

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modifications were discovered. The first identified SUMO target was RanGAP1, whose modification is exceptionally stable [19, 20]. Since then, a number of genetic and proteomic studies have been performed to identify sumoylation substrates in different species. These strategies uncovered between 100 and 300 proteins that can be modified by SUMO in *Saccharomyces cerevisiae* and in *Drosophila melanogaster* [21–25], and approximately a hundred target proteins of human SUMO1 and SUMO2 [26–29]. Functional categories of SUMO-modified proteins include SUMO conjugation system enzymes, chromatin- and gene silencing-related factors, DNA repair and genome stability proteins, stress-related proteins, transcription factors, proteins involved in translation and RNA processing, and a variety of metabolic enzymes [21–30].

The mechanism of sumoylation

Several excellent reviews are available on the enzymes and mechanisms involved in the SUMO pathway [10, 31–33], so we will only present a brief outline herein.

The sumoylation reaction leads to the formation of an isopeptide bond between a lysine residue in the acceptor protein and the C-terminal glycine residue of SUMO. The reaction requires the participation of an enzymatic cascade. SUMO is synthesized as an inactive molecule. In the first step, a SUMO hydrolase (Ubiquitin-like specific proteases (Ulps) in yeast or SENtrin-specific proteases (SENPs) in human) cleaves the C-terminus of the nascent SUMO to expose the di-glycine motif used for conjugation [11, 17]. Mature SUMO is subsequently activated in an ATP-dependent process by the E1-activating enzyme, which consists of two subunits: SUMO-activating enzyme 1 (SAE1), also known as Aos1 (Activation of Smt3p in yeast), and SUMO-activating enzyme 2 (SAE2), also known as Uba2 (Ubiquitin-like activating enzyme subunit 2 in yeast), which are involved with adenylation and thio-esterification functions, respectively [31]. In the next step, transesterification of a catalytic cysteine residue in the E2-conjugating enzyme UBC9 mediates the ligation of SUMO to the ε-amino group of a lysine side chain contained within a sumoylation consensus motif of substrate proteins. Consensus sites on SUMO-modified proteins are ψKX(D/E) (where ψ is a large hydrophobic residue, K is the above-mentioned lysine, X is any amino acid and D/E is an acidic residue). These residues directly interact with E2-UBC9, positioning the lysine within the E2-active site. The context of this consensus sequence within the substrate is critical for efficient interactions. Therefore, many SUMO consensus sites are found in extended loops or in regions outside the globular fold. Composite sequence motifs that include both SUMO consensus sequences and additional residues have also been identified [34]. Additional elements in these sequences allow the coupling of sumoylation to other post-translational modifications. A well-characterized example is the phosphorylation-dependent SUMO motif (PDSM), also known as the phospho-sumoyl switch, which is found in many proteins and possesses a phosphorylatable residue adjacent to the SUMO acceptor site, ψKX(D/E)XXSP. In these proteins, phosphorylation increases the levels of SUMO conjugation, thus converting phosphorylation signals into sumoylation-based actions. This mechanism affects the activity of different transcription factors and will be discussed in the context of its impact on postsynaptic dendritic morphogenesis. E2-substrate interactions are often sufficient to guarantee substrate specificity for sumoylation. However, in some cases, substrate specificity depends on non-covalent interactions that occur between the E2-SUMO thioester and SUMO-Interacting-Motifs (SIMs). SIMs consist of a short stretch of hydrophobic amino acids flanked by acidic residues. When present in SUMO substrates, SIMs can mediate SUMO modification, but they can also promote other kinds of protein interactions or influence protein localization [35]. SIMs are also a hallmark of a novel family of proteins, the small ubiquitin-related modifier (SUMO)-targeted ubiquitin ligases (STUbLs). STUbLs are ubiquitin ligases that appear to be recruited to sumoylated proteins and proteins containing SUMO-like domains to mediate their ubiquitination and subsequent desumoylation/degradation [36]. In some cases, sumoylation of specific targets requires SUMO E3-ligases, which help to confer specificity and accelerate catalysis [31]. The three best-characterized types of SUMO E3-ligases include the SAP and Miz1 domain proteins (SIZ1 and SIZ2) and the Protein Inhibitors of Activated STAT (PIAS), which constitute the SIZ/PIAS family, the nuclear pore complex-associated protein (RanBP2) and the Polycomb 2 homologue (PC2). E3-ligases act by regulating the interaction between the substrate proteins, SUMO, and UBC9 [10, 31, 33].

SUMO deconjugation is essential for regulation of the sumoylation pathway. The same family of Ulps/SENPs that participate in the processing of SUMO precursors also function to remove SUMO from proteins and to edit SUMO chains (cleavage of one or more SUMOs from a poly-SUMO chain) [37]. In humans, six SENPs have been identified. On the basis of cellular localization, substrate specificity and sequence homology, they are divided into three subfamilies. SENP1 and SENP2, which can deconjugate either SUMO1 or SUMO2/3-modified proteins, belong to the first subfamily. The second subfamily includes SENP3 and SENP5 and the third consists of SENP6 and SENP7; these two groups recognize SUMO2/3 as substrates.

Sumoylation can occur in different subcellular locations. While most SUMO conjugates are found in the nucleus,

accumulating evidence indicates the presence of sumoylation machinery in the nuclear and cytoplasmic compartments and in the plasma membrane [38–40]. Accordingly, a number of non-nuclear substrates have been identified, including integral membrane proteins such as G-coupled protein receptors [41], suggesting that SUMO can regulate events outside the nucleus.

Effects of SUMO modification on substrate function

Although many of the molecular mechanisms by which sumoylation alters the biological function of its substrates remain unknown, it is generally recognized that modified proteins experience a change on their interacting properties with their partners. At the cellular level, the outcomes of such changes can be diverse, but they converge on limited actions. First, protein activity can be altered, either increased by facilitation of binding to other proteins or DNA or decreased by masking binding sites. Second, different protein interactions with its traffic carriers can result on changes on subcellular localization, which in turn can be critical for accessing a target. Finally, sumoylation can lead to changes on protein stability through crosstalk with ubiquitination [42, 43]. Examples for each of these situations have been demonstrated both in cell culture and in vivo.

The overall biological importance of sumoylation during embryonic development remains unclear. However, given the enormous capacity of this reversible modification for modulating signal amplification, it is anticipated that a strong connection between sumoylation and embryonic development will be found. In support of this view, interesting examples of crucial roles of the SUMO pathway on the regulation of developmental processes have been described recently. In this article, we examine the role of SUMO in specific developmental processes, such as photoreceptor development, neural crest and inner ear specification, axis formation, hematopoiesis control, and cardiac development, with attention to the distinct mechanisms by which sumoylation regulates cell specification and patterning in these tissues.

General importance of sumoylation in embryonic development: in vivo functional studies with components of the SUMO pathway

Studies with mutant animals have demonstrated critical roles of the non-redundant SUMO pathway proteins in basic early processes of development, and mutations in redundant components of the pathway have revealed specific roles in regional patterning and cell-fate specification. The most significant findings are described in this section and are summarized in Table 1. In addition, the diverse developmental roles of sumoylation in mammals are depicted in Fig. 1.

UBC9, a unique E2-conjugating enzyme with functions in mitosis and morphogenesis

The SUMO E2 enzyme UBC9 is a single gene-encoded protein in the SUMO pathway. Studies of the loss of UBC9 function reflect the impact of sumoylation on entire organisms. Null or hypomorphic mutants for UBC9 have been reported for nearly all of the developmental models. Although different phenotypes have been described for different species, these studies have shown that UBC9 and by extension the SUMO pathway play essential roles from the very early stages of development. Its participation in various aspects of mitosis is at the center of these basic functions and has been demonstrated in several species from lower to higher eukaryotes. Such functions include chromosome integrity and segregation, cell cycle progression, kinetochore assembly, and function and cytokinesis. Initial evidence of these functions was obtained by studying yeast models, where impairment of components in the SUMO pathway results in significant defects in cell cycle progression and in chromosome segregation. In budding yeast, UBC9 is essential for degradation of B type cyclins and its loss causes the block of cell cycle progression at the G2/M phase [44]. Sumoylation is also needed for efficient proteolysis mediated by the anaphase-promoting complex/cyclosome (APC/C), as evidenced by cells depleted of UBC9 or SUMO (Smt3) being mostly arrested with undivided nuclei and with high levels of securin Pds1, an APC/C substrate [45]. In vertebrates, *Xenopus* egg extracts have been used as a model for testing the importance of sumoylation in the mitotic cell cycle. In these extracts, Topoisomerase-II was shown to be a significant sumoylation substrate during mitosis and is exclusively modified by SUMO2 [46]. Accumulation of unmodified Topoisomerase-II by inhibition of de novo SUMO conjugation, using a dominant-negative form of UBC9, blocks the dissociation of sister chromatids at the metaphase-anaphase transition, implicating SUMO2 conjugation of Topoisomerase-II in the remodeling of mitotic chromosomes at the metaphase–anaphase transition. Loss of *Ubc9* in mice leads to embryonic lethality during the early postimplantation stage and results in selective apoptosis of cells of the inner cell mass (ICM) in blastocysts [47]. In culture, mutant blastocysts also undergo apoptosis after 2 days and cells of these embryos present chromosome missegregation as well as major abnormalities in nuclear organization, such as disassembled nucleoli and promyelocytic leukemia (PML) nuclear bodies (Fig. 1). Likewise, reduction of maternal and zygotic UBC9 activity in zebrafish embryos causes early

Table 1 Roles of components of the SUMO pathway in embryonic development

Protein and activity	Species	Developmental phenotype (loss of function)	Molecular or cellular mechanism	Reference
UBC9 E2-conjugating	*S.* cerevisiae	Block of progression through mitosis	Degradation of S- and and M- phase cyclins	[44, 45]
	C. elegans	Embryonic arrest at gastrulation		[49]
	Drosophila	Patterning defects, posterior hole, mild dorsal closure defects (embryos) melanotic tumors (larvae)	Nuclear localization of Bicoid and Dorsal, Spalt-like and Medea proteins	[50–54]
	Zebrafish[a]	Early embryo apoptosis and defects in brain, eyes, and cartilage formation	Regulation of mitosis in chondrocytes	[48]
	Mouse KO	Early embryonic death, inner cell mass apoptosis	Regulation of chromosome segregation and nuclear integrity	[47]
SUMO	*Drosophila*	Defective oogenesis. Embryonic and larval lethality Defects in Ras signaling, cell cycle and pattern formation	Nuclear export-import of different transcription factors and regulation of cell cycle proteins	[22, 53]
	Xenopus[a]	Defective mesoderm formation, disruption of axis elongation	Involved in activin/nodal signaling	[68]
	Mouse KO	Dispensable for embryonic development		[63, 64]
PIAS (E3-ligase)				
SU(VAR)2-10	*Drosophila*	Larval–pupal lethality with melanotic tumors. Defective chromosomal structure and inheritance	Negative regulation of JAK/STAT. Chromosome establishment and maintenance	[69, 73]
PIASy	Mouse KO	Dispensable for embryonic development		[75, 76]
PIAS3	Mouse[b]	Photoreceptors adopt a cone-like morphology	Targets *Crx* and *Nr2e3* photoreceptor promoters Directs the correct expression of cone opsin genes	[105, 106]
PIASx	Mouse[b]	Reduced dendritic claw differentiation	Repression of MEF2A driven transcription	[78, 79]
	Mouse KO	Mildly disturbed spermatogenesis		[77]
SUMO-isopeptidases				
SENP1	Mouse KO	Fetal anemia, deficient survival of erythroid progenitors	Regulation of HIF1α stability	[87]
SENP2	Mouse KO	Abnormal trophoblast stem cell development, essential for G1/S transition in TS cells	Regulation of p53-MDM2 pathway	[89]
		Embryonic lethal at 10dpc, smaller cardiac chamber	Increased repressive activity of Polycomb	[88]

[a] Morpholino mediated inactivation; *KO* gene knockout

[b] Function inactivation in in vivo electroporation assays; otherwise, gene mutations in the whole organism or in germ line clones

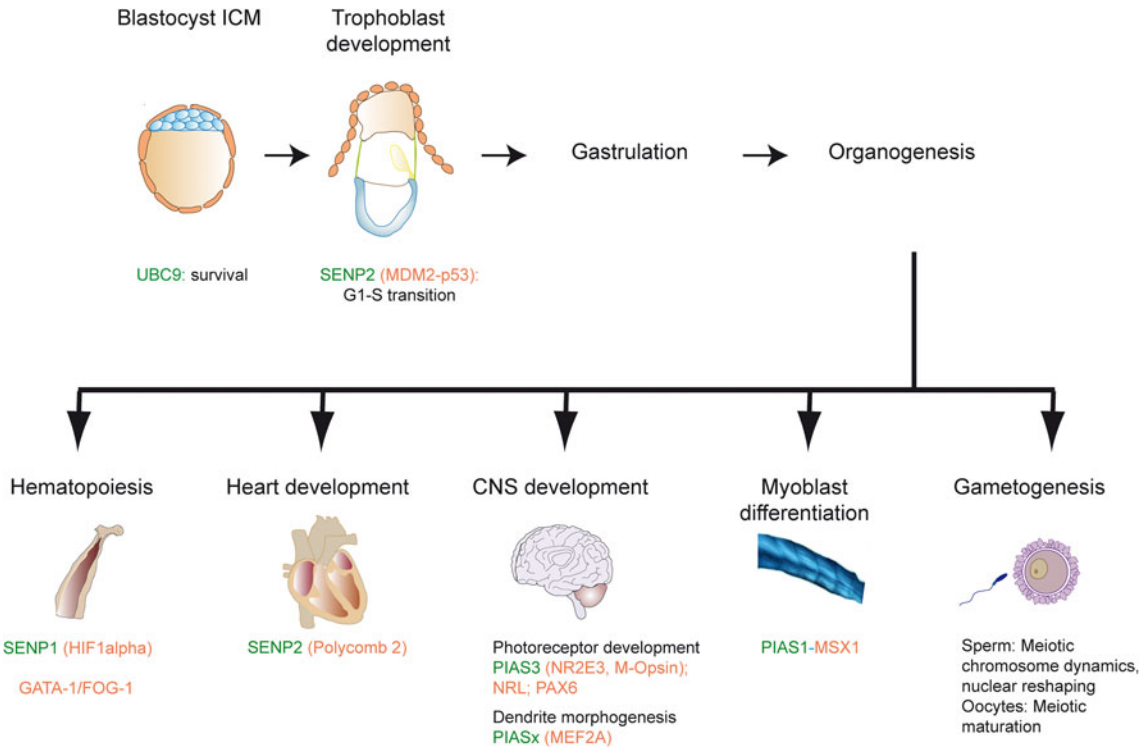

Fig. 1 Components of the SUMO pathway (in *green*) participate at distinct steps of mouse embryonic development from pre-implantation to organogenesis and gametogenesis. In some cases, their specific targets have been identified and are indicated in parentheses (in *orange*). Additional developmental factors whose sumoylation seems to be involved in the indicated developmental process are listed (in *orange*). In some cases, PIAS proteins interact with developmental factors independently of sumoylation. For example, PIAS1-Msx1 interaction is important for in vitro myoblast differentiation, but sumoylation is not involved on their actions. SUMO1 and other sumoylation proteins have very significant expression patterns during spermatogenesis, oogenesis, and meiosis. Functional results obtained from yeast and *Drosophila* support the notion that SUMO-conjugation has a role in gametogenesis

embryonic apoptosis, but inactivation of zygotic transcription of this gene in embryos with a maternal supply led to later specific developmental defects in the brain, eyes, and cranial cartilage [48]. Late proliferating cells of these tissues exhibit abnormalities in the cell cycle during the G2/M transition, as was observed for yeast where UBC9 is required for progression through mitosis [44].

Aside from its functions in mitosis, loss-of-function studies of UBC9 have revealed effects on morphogenetic processes. Knockdown experiments of *ubc9* in *Caenorhabditis elegans* (*C. elegans*) resulted in embryonic arrest after gastrulation. The lack of UBC9 also resulted in pleiotropic defects in larval development in animals that completed embryogenesis. The most common abnormality is associated with morphogenesis of the vulva [49]. In *Drosophila*, UBC9 is mainly nuclear and probably maternally provided and uniformly expressed during embryogenesis [50]. UBC9 is important to localize in the nucleus embryonic proteins such as Dorsal and Bicoid [50] or larval transcription factors such as Spalt-like proteins [51]. In the case of Dorsal, sumoylation overcomes its Cactus-mediated sequestration in the cytoplasm [52]. UBC9 is encoded in *Drosophila* by the gene *lesswright (lwr)*. Indeed, *lwr* null mutants (in regulatory alleles called *semushi*), are recessive lethal and present a posterior hole or a mild closure defect [53], or exhibit reduced numbers of thoracic and abdominal segments with patterning defects associated with the misregulation of the anterior-posterior morphogen Bicoid [54]. Flies with hypomorphic *lwr* mutations survive until the larval stages, at which they present melanotic tumors and overproduction of larval hemocytes [55]. High-level expression of UBC9 in the central nervous system in males causes lethality [56].

The SUMO proteins: essential and redundant functions

Invertebrate species have only one SUMO-encoding gene. For these organisms, complete inactivation of this gene leads to the expected lethal phenotypes. In *C. elegans*, a *smo-1 (sumo-1)* mutant allele with a maternal supply of wild-type SUMO shows 100% sterility [57]. Phenotypic analysis of this mutant demonstrated that SUMO is required for postembryonic development of the reproductive system and for gametogenesis. Neither mature sperm nor normal oocytes could be found in adult mutant homozygous animals. These authors also identified LIN-11

as a substrate for SUMO modification and established the importance of its sumoylation for uterine morphogenesis. Recent work uncovered other targets for sumoylation in *C. elegans* [58, 59], including some that are related to earlier steps of vulva development. For example, LIN-1, an ETS (erythroblast transformation-specific) domain transcriptional repressor that inhibits vulval cell fates, was shown to be sumoylated. The authors found that this modification promotes transcriptional repression activity of LIN-1 and mediates its association with the NuRD transcriptional repression complex [58]. In addition, components of the SUMO pathway were identified in a genome-wide screen for genes that produce multivulval phenotypes. In this work, evidence suggested that sumoylation functions both to antagonize Ras-induced vulval development by modulating the activity of repressors of transcription and to inhibit Notch signaling, one of the pathways implicated in the development of the gonad and germ line [59]. In *Drosophila*, SUMO is encoded by a single gene, *smt3*. SUMO and the enzymes involved in the pathway are particularly enriched during early stages of development. SUMO is maternally inherited and accumulates in preblastoderm embryos, appearing uniformly distributed throughout the embryo at the cellular blastoderm and gastrulation stages [60]. It is present in the nuclei during embryogenesis, even at interphase in postgastrulation embryos, and appears to localize in the chromosomes during mitosis [61]. Later, *smt3* mRNA accumulates preferentially in the CNS and in the gonads. Flies homozygous for *smt3* hypomorphic alleles exhibit a five-fold decrease of *smt3* transcript and present a lethal period before or during the early second larval instar. Analyses of germ line clones with hypomorphic *smt3* revealed that the maternal SUMO contribution is essential for embryo viability [22]. In *Drosophila*, maternal deposited RNA and proteins drive the early stages of zygote development. Thus, it is noticeable that the lack of maternal SUMO cannot be rescued by SUMO expressed from the zygote's genome, meaning that SUMO functions are necessary before the onset of zygotic transcription. Less than 30% of embryos derived from germ-line clones defective for SUMO function hatched and died during the first larval instar. The majority of the unhatched embryos died prior to cuticle formation, and the rest presented patterning defects. Half of these embryos presented diverse phenotypes of nuclear cycle defects affecting interphase chromosome structure, ploidy, chromatin condensation and loss of synchronization among others, implicating sumoylation in multiple events of mitosis [22].

Although SUMO paralogs in vertebrates share common conjugation properties, they also exhibit specificities, such as preferential conjugation to different substrates or different patterns of subcellular distribution, under physiological conditions. Accordingly, knockouts of SUMO genes in different species present specific phenotypes. Three SUMO paralogs are found in zebrafish. Morpholino-mediated inactivation of single paralogs or pairwise loss of *sumo1* and *sumo2* was shown to be compatible with normal development, whereas loss of all three *sumo* genes led to later developmental defects similar to those observed in the zygotic UBC9 knockdown, including reduced sizes of the brain and eyes, malformations in the jaw, and increased apoptosis in cells of the head region [62]. These results suggested significant functional redundancy between SUMO paralogs. Experiments with SUMO1 knockout mice also support these findings. Two independent studies indicated that SUMO1 is not essential for mouse development and that in vivo functions of this protein can be substituted by other SUMO paralogs [63, 64]. In both reports, compensatory sumoylation of RanGAP1 by SUMO2/3 was demonstrated. Redundancy apparently serves to guarantee minimal levels of sumoylation of key proteins such as RanGAP1. However, studies analyzing the ability of SUMO1 to compensate for SUMO2/3 lack of function have not been reported.

Contrary to the above-described results, Alkuraya et al. reported craniofacial defects in heterozygous embryos from a *SUMO1* mouse line containing a β-galactosidase gene trap vector [65]. This phenotype was correlated with cleft lip and palate defects observed in a patient with *SUMO1* haploinsufficiency. Moreover, additional studies identified other cleft palate genes such as *Tbx2*, *Eya1*, and *Msx1* as sumoylation substrates [65–67]. Altogether, these data implicated SUMO1 in palatogenesis. However, these results could not be reproduced by either of the SUMO1 knockout studies described earlier [63, 64]. Therefore, the differences could be explained by the genetic backgrounds or by the targeting strategy used by Alkuraya and colleagues, as other genes may have also been influenced.

Single knockouts of SUMO paralogs in *Xenopus* helped to discover specific non-redundant participation of sumoylation in signaling pathways. For example, blocking the translation of SUMO1 in frogs caused disruption of axis formation and produced a phenotype resembling that produced by injection of activin/nodal inhibitors, indicating the participation of XSUMO1 in activin/nodal signal transduction and mesoderm induction [68].

Specific roles of PIAS proteins: SUMO E3-ligase-dependent mechanisms

PIAS proteins have been shown to interact with DNA via an N-terminal region called the SAP (saf-A/B, acinus and PIAS) domain. Furthermore, they do not operate only as E3-ligases, as their co-regulatory effects sometimes depend exclusively on their ability to interact with DNA. Thus, PIAS proteins appear to be involved in an important mechanism for transcriptional regulation. In *Drosophila*, the PIAS

proteins are represented by several SU(VAR)2-10 isoforms encoded by a single gene, *Su(var)2-10* [69]. As in mammals, these proteins are negative regulators of the JAK/STAT pathway, which controls different developmental processes in *Drosophila*, such as border cell migration during oogenesis or blood cell and eye development probably through negative regulation of the transcription factor Stat92E [60]. The *Su(var)2-10* gene was initially identified as a suppressor of position effect variegation, a phenotype that has been related to heterochromatin-I-induced gene silencing. Analyses of *Su(var)2-10* mutant flies showed that SU(VAR)2-10 protein functions are involved in chromosomal structure and inheritance. Flies with different combinations of *Su(var)2-10* mutations die as late larvae or early pupae with melanotic tumors and present aberrant telomere clustering and association with the nuclear lamina [69]. The presence of melanotic tumors is an interesting phenotype often observed in *Drosophila* sumoylation mutants such as the previously described *lwr* mutant (UBC9 section). In some cases, the formation of larval melanotic masses occurs during the immune response and is mediated by hemocytes [70]. Innate immune responses and hematopoiesis in eukaryotes are linked by Rel-related proteins and Toll signaling [71]. The *Drosophila* Rel-related protein Dorsal is essential for dorsoventral embryonic axis formation and is part of the Toll signaling pathway. Sumoylation involving Dorsal has been related to the immune response and to hemocyte production. Dorsal sumoylation promotes Dorsal nuclear uptake and is associated with the potentiation of the immune response in S2 culture cells and in first instar larvae [52]. By contrast, Huang et al. (2005) found that defective *lwr* larvae present high amounts of Dorsal in the nucleus, leading to overproduction of hemocytes. In addition, mutations in *dorsal* and in *Dorsal-related immunity factor*, a gene encoding for another Rel-related factor Dif, suppress the effects of *lwr* mutations [72]. Three signal transduction pathways influence the number of hemocytes in circulation: JAK/STAT, Ras and Toll/Cactus pathways. Members of these important pathways such as Stat92 [73], Ras and Dorsal [22] are SUMO targets in *Drosophila*. Therefore, additional studies are needed to better understand the roles and consequences of sumoylation in all of the processes involving Dorsal and other Rel-related factors.

PIASy was identified in *Xenopus* egg extracts as the primary E3-like factor involved in the mitotic modification of Topoisomerase-II by SUMO-2 conjugation [74]. Immunodepletion of PIASy from egg extracts abolished this modification and failed to recruit UBC9 to mitotic chromatin. In mammals, the PIAS family consists of at least five genes and/or spliced variants (PIAS1, PIAS3, PIASy, PIASxα, PIASxβ). Among the components of the SUMO pathway, this family of proteins has the most diversity. Some of the PIAS genes were shown to exhibit specific gene expression patterns during development. Therefore, the specificity of the sumoylation pathway possibly resides, at least partly, in the non-redundant actions of the PIAS proteins.

Two different studies reported the characterization of PIASy-deficient mice [75, 76]. These analyses demonstrated that PIASy is not essential for embryogenesis or adult life. Mutant embryos showed no significant phenotypic abnormalities, except for a modest reduction of signaling in response to IFN-γ and Wnt agonists. These studies suggested that PIASy also acts redundantly with other PIAS proteins.

PIASx exhibits an interesting pattern of expression. It is highly expressed in testis, spermatogonia, pachytene spermatocytes, and Sertoli cells, and thus has been associated with having a role in testicular function. The *Piasx* gene was disrupted in mice, leading to reduction in the testis weight and sperm count. However, the quality of their sperm was normal and fertility was not affected, indicating that PIASx is not essential for spermatogenesis [77]. These mice clearly presented defective spermatogenesis because a high number of apoptotic testicular cells were found; however, this deficiency seems to be rescued by other PIAS proteins. Fully inactivating the SUMO pathway during testicular development would be interesting. A different approach to analyze the function of PIASx in cerebellar granule neurons uncovered a role of this protein in postsynaptic morphogenesis of dendrites, a fundamental process in synapse formation. A critical transcription factor for the differentiation of dendrites is myocyte enhancer factor 2A (MEF2A). A SUMO-modified form of MEF2A with transcriptional repression function was shown to induce postsynaptic dendritic differentiation [78]. In addition, PIASx was identified as the specific SUMO E3-ligase that regulates MEF2A transcriptional activity in the cerebellar cortex [79]. The essential role of PIASx for dendritic differentiation was demonstrated by gain-of-function and genetic knockdown experiments in rat cerebellar slices and in the postnatal cerebellum in vivo (Fig. 1). In these models, sumoylation of MEF2A by PIASx represses MEF2-dependent transcription and promotes dendritic claw differentiation. This work is particularly important in the context of phosphorylation-dependent SUMO conjugation, given that the sumoylation site in MEF2A is a PDSM. The biological significance of phosphorylation and sumoylation coupling has been debated based on these results and further data from other groups. Additional studies have demonstrated that phosphorylation of MEF2A at a serine residue prevents activation of MEF2 target genes, which ultimately mediate synapse disassembly [78]. On the other hand, calcium influx activates the phosphatase calcineurin, which in turn reverses the phosphorylation [78, 80, 81]. The authors suggested that sumoylation acts as a

switch that converts MEF2A from a transcriptional activator to a transcriptional repressor. In the absence of calcium signaling, MEF2A is phosphorylated and sumoylated, leading to repression of MEF2A target genes and promotion of synapse formation and maturation. Calcium influx triggered by neuronal activity causes dephosphorylation of MEF2A, resulting in desumoylation and synapse disassembly.

PIAS3 presents a particularly interesting expression pattern in the mouse retina, encouraging different analyses of PIAS3 function during photoreceptor development. The results from independent studies currently support a critical role for this E3-ligase in the transcriptional regulation of rod- and cone-specific genes. These studies will be discussed in the context of cell-fate decisions.

PIAS proteins in development: SUMO-ligase-independent functions

Several instances have been reported in which the effect of PIAS proteins on substrate function is uncoupled from the SUMO ligase activity against a particular substrate. For example, PIAS proteins often operate through the relocalization of transcriptional regulators to different subnuclear compartments. This relocalization has been shown to be determinant for the binding of transcription factors to target promoters and is usually involved in transcriptional repression. Lee et al. [66] illustrated this mechanism in mammals, showing that PIAS1 confers DNA-binding specificity on the MSX1 homeoprotein and that this activity contributes to preventing terminal differentiation of myoblast precursors. MSX homeobox genes play key roles in regulating cellular differentiation during development. All three members of this family have been characterized for their expression in progenitor populations, where they seem to function by preventing terminal differentiation [82]. MSX1 inhibits myoblast differentiation through repression of MyoD and Myf5, two myogenic regulatory genes [83]. Repression of MyoD involves the binding of MSX1 to a core enhancer region (CER), which is located in the MyoD promoter [84]. PIAS1 was identified in a search for MSX1-interacting proteins that cooperate in vivo with MSX1 for binding to specific DNA regions [66]. PIAS1 interacts with MSX1, leading to its modification by sumoylation. By comparing the transcriptional activity of exogenously expressed MSX1 with that of truncated or mutated derivatives in myoblast cells and by analyzing the differentiation properties of transfected cells, PIAS1 was shown to be required for conferring binding specificity of MSX1 to CER and consequently for repression of MyoD as well as for differentiation (Fig. 1). However, these experiments indicated that sumoylation of MSX1 was not necessary for any of these activities. Further work involving ChIP assays, immunofluorescent co-localizations, and fluorescence resonance energy transfer (FRET) showed that the interaction of MSX1 with PIAS is sufficient for the appropriate localization and retention of MSX1 at the nuclear periphery of myoblast cells. However, it remains unclear how this interaction promotes MSX1 binding to a particular site in vivo. Accordingly, the same authors found that myogenic regulatory genes targeted by MSX1 were also located at the nuclear periphery in undifferentiated muscle cells, where they are inactive, but toward the center of the nucleus in differentiated muscle cells, where repression is lost. However, MSX1-PIAS1 interaction is not involved in this reorganization of MSX1 target genes. This evidence illustrates a sumoylation-independent mechanism in which a co-factor can help a homeoprotein to increase its DNA-binding specificity by regulating its localization to the proximity of target genes.

XPIASy was identified in *Xenopus* to interact with XSmad2 in a yeast two-hybrid screen [85]. Through gain- and loss-of-function approaches, XPIASy was shown to negatively regulate the transcription activity of XSmad2 during mesoderm induction by a direct interaction. Although XPIASy was able to enhance sumoylation of XSmad2, this activity was not required for its transcriptional repression. These findings indicated that sumoylation-independent actions of a PIAS protein, as a co-regulator of transcription, are important for mesoderm formation in *Xenopus* development.

SENPs: central regulators of the sumoylation levels of developmental factors

Deconjugation of SUMO is likely to represent a key regulatory mechanism for sumoylation. As for the PIAS proteins, desumoylation enzymes in vertebrates constitute a complex family in which members exhibit differences in intracellular distribution and substrate specificities. However, in contrast to the subtle phenotypes yielded by the PIAS loss-of-function studies, the SENP knockout mouse embryos reported to date do not survive to birth, suggesting that the SENPs are not redundant and must have specific substrate specificity during development.

The first description of a mutation for a mammalian SENP gene was a retroviral insertion in the mouse gene for SENP1 (*SuPr-2*), which led to a reduced expression of this gene [86]. Analysis of this mutation revealed that the SENP1 deficiency causes a significant increase in the overall level of conjugation with SUMO1 but did not affect the SUMO2/3 protein sumoylation levels, indicating that SENP1 does not play an important role in SUMO2/3 deconjugation during development. Mutant mice presented phenotypic defects in the placenta that were incompatible

with embryonic viability and produced death after midgestation (12.5–14.5 dpc). Although a general participation of SENP1 in development was demonstrated in this work, the targets underlying the mutant phenotype were not identified and the specific role of SENP1 was not precisely defined. Another transgenic mouse with complete inactivation of SENP1 function was recently reported [87]. These mice presented hematopoietic defects, providing evidence for a role of SENP1 in the hypoxic response through the regulation of the hypoxia-inducible factor 1 alpha (HIF1α) stability (see Sect. Control of protein stability by sumoylation). In addition, two different SENP2 null alleles were reported, indicating a role of this enzyme in heart development [88] and in cell cycle control during trophoblast development [89]. These works are discussed below.

In vivo functional studies involving sumoylated developmental genes

Several developmental regulators have been identified as SUMO targets or as proteins that interact with components of the SUMO pathway. Some have very well-defined roles in development, but the influence of sumoylation on their activity in vivo has not yet been described. Examples of such factors are PROX1, the master regulator for the development of lymphatic vasculature [90]; PAX6, the critical transcription factor for eye and brain development [91] and the homeodomain-interacting protein kinases (HIPK 1 and 2), which are implicated in the phosphorylation and repression of homeodomain-containing transcription factors [92–94]. At the same time, compelling data showing the consequences of the SUMO modification on important developmental proteins has been published in recent years. Evidence for clear roles of sumoylation on developmental processes in living organisms or in well-characterized differentiation systems is described below and summarized in Table 2.

Control of cellular fate by sumoylation

Cellular decision-making is the process whereby cells assume a different and heritable fate and is one of the key events underlying development. Recent studies illustrate the involvement of sumoylation in such decisions. In this section, we consider examples in three different species, from worms to mammals, in which SUMO conjugation provides instructions for a cell differentiation pathway. Although the molecular mechanisms are not defined in any of these cases, the available evidence indicates that sumoylation operates in different way, such as by converting a transcription factor from an activator to a repressor, differential recruitment of activators and repressors, or altering subcellular localization.

One of these mechanisms occurs in defining neural crest *versus* inner ear development in *Xenopus*, a process in which SOXE proteins have a determinant role. SOX proteins are a family of transcription factors whose hallmark is the DNA-binding motif called the HMG (high mobility group) domain. The SOX group E consists of SOX8, SOX9, and SOX10. These transcription factors contain an additional DNA-dependent dimerization domain, which is unique among SOX proteins [95]. SOXE genes have been extensively studied for their role in various developmental processes including neural crest differentiation.

Neural crest cells are a population of migratory stem cells in vertebrates. They populate diverse regions throughout the embryo and give rise to a wide range of derivatives that include most of the neurons and glia of the peripheral nervous system, melanocytes, and craniofacial cartilage [96]. In *Xenopus*, SOX9 and SOX10 are expressed in neural crest precursor cells and are known to drive the formation of multiple neural crest derivatives [97, 98].

Table 2 Effects of sumoylation on developmental regulators during embryonic development

SUMO target	Species	Related developmental process	Consequence of sumoylation	References
Dorsal	*Drosophila*	Embryonic patterning	Nuclear import	[50]
Medea (Smad4)	*Drosophila*	Amnioserosa and dorsal epidermis cell fates	Restricts signaling rate of Dpp	[53]
SCM	*Drosophila*	HOX silencing	Impedes SCM silencing function	[128]
SOXE: 9 and 10	*Xenopus*	Neural crest and inner ear development	Modulates the activity of Sox9 and Sox10 on neural crest and inner ear development	[102]
GATA1-FOG	Murine cell line	Hematopoiesis control	Proper localization of FOG1-dependent GATA1 targets	[109]
TBX-2	*C. elegans*	Development of anterior pharyngeal muscles	Affects nuclear localization of TBX2	[67]
SOP-2	*C. elegans*	HOX silencing	SOP-2 location in nuclear bodies, allows HOX silencing	[127]
p63α	Zebrafish	Neural dorsoventral pattern	Regulates stability of deltap63α	[119, 120]

Parallel studies have also indicated the participation of SOXE factors in inner ear development and identified SOX9 as a typical marker of the otic placode [99].

Initial experiments in *Xenopus* embryos indicated that both SOX9 and SOX10 were capable of inducing an expansion in the neural crest progenitor domain [100, 101], suggesting that they had equivalent activities. However, late expression of SOX9 and SOX10 is restricted to specific neural crest-derived populations, implying that they may also exhibit distinct activities [101]. Because these two SOXE factors participate in divergent developmental functions in cells where their expression overlaps, a better understanding of the mechanisms underlying their functional diversity was needed. To address this question, Taylor and LaBonne [102] conducted a two-hybrid screen that led to the identification of SUMO and UBC9 as SOXE-interacting proteins. Through biochemical studies, they demonstrated that both SOX proteins were sumoylated in *Xenopus* embryos. Based on these findings, they constructed a SOX9 mutant that cannot be sumoylated and a SOX9-SUMO1 fusion that mimics the effect of constitutive SUMO conjugation due to its covalent attachment to SUMO. By injecting these constructs into embryos, they discovered that each of these variants had distinct effects on neural crest and otic placode formation. Whereas constitutively sumoylated SOX9 increased the size of the otic placode and the expression of markers of inner ear development, the non-sumoylatable SOX9 variant caused a reduction of this region while favoring the expression of markers of neural crest and its derivatives. They concluded that the sumoylation state of SOXE factors modulates their function and determines their final action as mediators of neural crest or otic placode formation. This study illustrated a mechanism of diversification of function for conserved proteins by posttranslational modification. Identifying the partners and target promoters that are sensitive to the sumoylation state of SOXE factors is crucial.

The vertebrate retina is another model suitable for the study of cell-fate decisions. Retinal photoreceptors are comprised of rod and cone subtypes, which are derived from a common progenitor but differ in function and gene expression. CRX and NR2E3 are two transcription factors that are co-expressed in rod photoreceptors and their precursors and play a central role in their formation. NR2E3 is a dual-function transcriptional regulator that exerts opposing effects on the transcription of rod *versus* cone genes, promoting the expression of the former while repressing the expression of the latter [103]. *Pias3* mRNA is also selectively expressed in developing photoreceptors within the mouse retina [104]. This observation led to an analysis of PIAS3 function during rod photoreceptor development [105]. In vivo overexpression of PIAS3 in developing retina revealed that it promotes rod differentiation and represses expression of cone-specific genes in rods (Fig. 1). These authors found that PIAS3 binds NR2E3 and CRX and further demonstrated that it could be found together with these transcription factors on the promoters of photoreceptor-specific genes. They also showed that PIAS3 catalyzed sumoylation of NR2E3. By testing mutant forms of PIAS3 in which the E3-ligase activity was turned down, NR2E3 sumoylation was shown to be required for repression of cone-specific genes. Finally, double chromatin immunoprecipitation (ChIP) assays revealed that the promoter regions of both rod- and cone-specific genes were hypersumoylated compared to genes not expressed in the retina. This last finding was intriguing and suggested additional functions for PIAS3-mediated sumoylation during retina development. More recent work by the same group of researchers shed light on this mystery [106]. They found that PIAS3 also acts in cone photoreceptors to direct the correct expression of distinct subtypes of cone opsin genes that are essential for color vision. PIAS3-dependent sumoylation results in activation of expression of the M-opsin subtype (Opn1mw), which is maximally sensitive to medium wavelengths, and leads to repression the S opsin subtype (Opn1sw), which is sensitive to short wavelengths. These data revealed a conserved function of PIAS3 in photoreceptor subtype specification and established for the first time a direct link between sumoylation and neuronal cell-fate specification. Another study that analyzed the function of NRL (another key regulator of rod *versus* cone photoreceptor cell fate) further supported the importance of SUMO conjugation for photoreceptor development [107]. NRL is sumoylated in vivo and this modification modulates its transcriptional activity on rhodopsin and *Nr2e3* promoters (Fig. 1). Through in vivo electroporation assays of *Nrl* null mice retinas, the authors demonstrated that NRL sumoylation is required for normal rod differentiation. In contrast to the findings by Onishi and colleagues regarding NR2E3 [105], PIAS3 appeared not to be involved in sumoylation of NRL, suggesting that another E3-ligase may be involved in fine-tuning NRL activity.

C. elegans is an excellent model system for studying cell specification. Embryo development in this species is mostly autonomous, although cell–cell interactions lead some cells to certain fates by breaking inherent symmetries. Studies of cell-fate specification in the worm have focused primarily on two classes of specification events. The first class consists of events that specify the six founder cells (AB, MS, E, C, D, and P), and the second type includes those that specify tissue types, irrespective of cell lineage. Loss of tissue identity factors causes either the formation of another tissue type or a loss of tissue integrity. TBX-2 is among the identity factors required for the development of the anterior pharyngeal muscles. These

muscles are descendants of one early embryonic blastomere termed ABa, and their fate is induced in the ABa cell lineage by GLP-1/Notch receptor activation. In *tbx-2* mutants, most or all ABa-derived pharyngeal muscles are lost, whereas the mesoderm-derived pharyngeal muscles are retained.

TBX2 is a T-box transcription factor. These factors encode an evolutionarily conserved 180- to 200-residue DNA binding domain and have important functions in cell fate and organogenesis. Recently, TBX2 was shown to interact with UBC9 and the E3 SUMO ligase GEI-17 in a yeast two-hybrid assay [67]. These authors found that TBX2 contains two consensus sumoylation sites and that RNAi-mediated loss of function of the sumoylation pathway components produces pharyngeal defects that resemble those in *tbx2* mutants. These results suggested the participation of sumoylation in pharyngeal development. In addition, normal nuclear localization of full-length TBX2 was shown to require *ubc9* and TBX2-GFP is predominantly distributed uniformly in early embryonic and body wall muscle nuclei in wild-type animals. By contrast, TBX2-GFP is strongly localized to nuclear puncta in *ubc9* (RNAi) animals. Thus, sumoylation appears to be involved in promoting localization of a transcription factor to a specific nuclear site in this case. One of the TBX2 sumoylation sites is located in the T-box and is highly conserved in vertebrate TBX2 subfamily members [108], suggesting that sumoylation is a conserved mechanism regulating the TBX2 activity.

SUMO-conjugation regulates the spatial organization within the cell

The location of macromolecules within a cell or nucleus affects their function and interactions with partners. Different mechanisms operate to determine the spatial organization of proteins and genes. Dynamic changes occurring in the cell include nucleocytoplasmic shuttling of signaling proteins, the association of regulatory factors to specific nuclear compartments and relocalization of genetic *loci* into particular subnuclear territories. All of these events are known to impact developmental processes and are strictly regulated. Recent discoveries suggest the participation of sumoylation within these processes. A study by Lee et al. provided new insights into how a master regulator of differentiation can exert different functions in distinct genetic *loci* [109]. These authors discovered that sumoylation dictates this differential behavior by influencing both the nuclear localization of the regulator and changes in the subnuclear positioning of particular *loci*. Their study was based on the master regulator of hematopoiesis GATA1, which is a transcription factor that activates or represses target genes essential for the control of leukemogenesis [110]. GATA1 can act independently or together with a coregulator known as friend of GATA1 (FOG1) depending upon the *locus* of a target gene [111, 112]. Specific GATA1 target genes are coregulated by FOG1 (e.g., *β-globin, α-globin* and *Slc4a1*) and others are controlled by an independent mechanism (e.g., *Epb4.9, Fog1,* and *Eklf*) [110, 112]. The functional importance of the GATA1 N-terminus was previously determined [113, 114]. A detailed analysis of significant residues in this region led to the identification of K137 as a sumoylation site [113]. To assess the function of sumoylation and the importance of K137 in the control of hematopoiesis, Lee and coworkers [109] used genetic complementation in a GATA1 null G1E cell line, which was able to recapitulate a normal window of erythropoiesis when GATA1 function was provided. They generated stable cell lines with *β*-estradiol-inducible expression of ER-GATA1 or ER-GATA1 with a point mutation in K137. Unlike ER-GATA1, ER-GATA1(K137) was incapable of inducing erythroid maturation. The authors found that certain GATA1 target genes were sensitive to the K137 mutation, whereas others were not. Sensitive genes turned out to be predominantly located at FOG-dependent *loci*, whereas insensitive genes were located at FOG-independent *loci*. Based on the observation that disruption of the sumoylation consensus sequence inhibited both GATA1 transcriptional activation and repression of FOG-dependent genes and tethering SUMO1 to a K137 mutant rescued it, the authors concluded that K137 sumoylation is a crucial molecular switch to control GATA1 function for an important subset of its target genes (Fig. 1). Quantitative ChIP analysis indicated that K137 and V205 mutations mediate FOG1 binding and decreased chromatin occupancy at select *loci* and diminished FOG1-dependent gene expression. However, sumoylation is independent of FOG1 and has no influence on FOG1 binding to GATA. Exploring whether the subnuclear localization of endogenous GATA1 could account for the different effects observed on target-genes, they found that the GATA1 K137R mutant presented an aberrant subnuclear localization. In order to establish the topographic relationship between GATA1 and its target genes, they performed 3D immuno-FISH experiments to measure the localization of FOG1/sumoylation-dependent and -independent targets relative to the nuclear periphery in G1E cells before and after expression of ER-GATA1 or G1E-ER-GATA1 (K137R). Through these experiments, they demonstrated that upon ER-GATA1 induction, FOG1/sumoylation-dependent genes migrated away from the nuclear periphery, whereas FOG1/sumoylation-independent genes persisted at the periphery. By contrast, expression of ER-GATA (K137R), which is incapable of inducing erythroid maturation, did not lead to relocalization of the FOG1/sumoylation-dependent *loci* from the

nuclear periphery to internal compartments. This remarkable observation indicated that GATA1 sumoylation somehow participates in the movement of FOG/sumoylation–dependent *loci*.

Nucleocytoplasmic shuttling of signaling molecules enables the cell to continuously monitor signal strength and provides the flexibility that is needed to react appropriately to changing signal intensities, which may be especially important during embryonic development when signals are highly dynamic. Shuttling of the Smads between the cytoplasm and nucleus in cells stimulated with TGFβ is well established. Mechanisms mediating Smad nuclear import and export have been extensively studied. Evidence recently obtained in *Drosophila* highlighted the participation of sumoylation in the nuclear export of one of the Smads. The *Drosophila* TGF-β/BMP signaling molecule Dpp functions in an embryonic cascade pathway to establish amnioserosa and dorsal epidermis cell fates through the action of the Smad transcription factors mothers against Dpp (Mad) and Med. Different events in the cascade lead to the phosphorylation of Mad (pMad), which then interacts with Med, and together they enter the nucleus to activate different gene targets according to specific Dpp concentration thresholds. Med is the ortholog of vertebrate Smad4, and both have been shown to constitutively shuttle between the nucleus and cytoplasm in a signal-independent manner [115, 116]. Miles et al. [53] demonstrated an important role of sumoylation in the Dpp (TGFβ/BMP) pathway, discovering that SUMO modification of the Smad transcription factor Medea (Med) promotes its nuclear export and thus restricts the range of action of Dpp signaling.

The relationship to sumoylation was revealed when the effectiveness of the Dpp pathway was evaluated in sumoylation-compromised flies. In *lwr* embryos with reduced levels of maternal and zygotic UBC9 activity, the expression patterns of different Dpp threshold responses and the duration of the signal are expanded. On the other hand, the embryonic lethality caused by a weak hypomorphic *dpp* allele is alleviated in *smt3* or *lwr* backgrounds. Interestingly, a portion of these *lwr* mutant embryos presented a phenotype of a posterior hole similar to that described for ectopic Dpp signaling in late-stage embryos. SUMO overexpression reduced the expression of Dpp-target genes, showing that increased sumoylation reduces Dpp signaling [53].

As expected for a sumoylation target, Med physically interacts with UBC9 in two-hybrid assays and is sumoylated in vitro at three residues, K113, K159, and K222. Two of these residues (K113 and K159) are the same as those that are sumoylated in Smad4, its ortholog in vertebrates. Med is also sumoylated in vivo in wild-type embryos but not in *lwr* embryos; consequently, non-sumoylatable Med phenocopies the *lwr* mutant phenotype increasing Dpp signaling range. Sumoylation of Med occurs in the nucleus and promotes Med export, thereby regulating the amount of the transcriptionally active nuclear pMad-Med complex [53].

Altogether, the data show that Med is the major SUMO target in the Dpp pathway and that Med sumoylation downregulates Dpp-responsive transcription in the early embryo. This study revealed an important role of sumoylation in refining the positional information generated by activation of a general developmental pathway, thereby regulating an intracellular transducer in vivo [53]. The authors suggested that Med sumoylation is a mechanism to ensure that Dpp-dependent transcription happens only as a response of continuous Dpp signaling. Sumoylated Med is exported from the nucleus, and although this mechanism may be wasteful in terms of signaling (Dpp), it confers protection against inappropriate activity, which may be catastrophic as Dpp and other morphogens are very potent in inducing different cell fates.

Control of protein stability by sumoylation

Unlike ubiquitination, sumoylation was until recently thought not to promote target protein degradation and could in fact stabilize targets by antagonizing their ubiquitination. Currently, it has become apparent that the two modification systems are not simply antagonistic but often communicate and jointly affect the properties of common substrate proteins, in some cases by being targeted to the same site. Numerous studies have linked ubiquitin-dependent proteasomal degradation of transcription factors to their transactivation potential. Some of these transcription factors are relevant for differentiation and development and have been reported to serve as substrates for both ubiquitination and sumoylation. Therefore, SUMO-directed ubiquitination has emerged as a potential developmental mechanism.

Recent studies identified crosstalk between the sumoylation and ubiquitination pathways in the control of HIF-1α. This connection became evident through the analysis of SENP1 null embryos [87]. These mice presented severe fetal anemia and deficient erythropoietin (EPO) production. SENP1 null embryos had more than 75% fewer erythrocytes than their wild-type littermates at E15.5 and died between days 13 and 15 of gestation (Fig. 1). These hematopoietic defects resembled those observed in *Epo* null embryos, in agreement with a deficiency in EPO production. In this work, the authors succeeded to uncover the specific role of SENP1 in the hypoxic response. HIF1α is regulated at the level of protein stability. During normal oxygen conditions, hydroxylation of proline residues by oxygen-sensitive enzymes promotes HIF1α binding to the

Von Hippel-Lindau (VHL) ubiquitin E3-ligase complex, leading to ubiquitination and subsequent degradation by the proteasome (in the cytosol). During hypoxia, proline hydroxylation is not efficient, and thus HIF1α escapes binding and translocates to the nucleus. In the nucleus, HIF1α is sumoylated and SUMO-conjugated HIF1α binds to VHL and the ubiquitin complex in a hydroxyl-proline-independent manner, thereby being marked for degradation. SENP1 is responsible for the deconjugation of sumoylated HIF1α in vivo. When present, sumoylated HIF1α is deconjugated and stabilized. As a consequence, unmodified HIF1α escapes VHL/proteasome degradation and activates gene expression of hypoxia-responsive genes. In SENP1$^{-/-}$ embryos, degradation of sumoylated HIF1α results in decreased EPO production and the observed phenotypes.

Another example of SUMO-directed ubiquitination that impacts development was discovered through functional studies of the transcription factor p63, a member of the p53 family. The p63 protein has the same transactivation, DNA-binding and oligomerization domains as p53, but also has a C-terminal extension containing a sterile α domain (SAM), which is characteristic for proteins that control developmental programs. Two transcription start sites combined with alternative splicing at the 3′ end generate several different transcripts of p63 that vary in their N- and C-termini. Mutations in p63 have been associated with developmental disorders in humans including limb malformations, ectodermal dysplasia, and facial clefts [117]. Some of the preferential sites for these mutations specifically target the so-called α isoforms of p63, directly implicating these variants in development. Extensive evidence demonstrated that p53 is both sumoylated and ubiquitinated [118]. These posttranslational modifications regulate the level of p53 and control its function. Similar to p53, p63 is also modified by these same peptides, but the functional implications of such modifications were not clear until recently. Ghioni et al. [117] identified a lysine residue (K637) in the SAM domain of human p63α as the single SUMO attachment site. This site is found in a previously described transcriptional inhibitory domain. They also showed that non-sumoylatable mutants exhibited a much stronger activation potential than its wild-type counterpart, indicating that SUMO conjugation regulates p63α transcriptional activity. While SUMO attachment potentiated p63α proteasomal degradation, it did not affect its intracellular localization. Further studies conducted in zebrafish helped to elucidate a specific developmental process in which sumoylation of deltaNp63α is determinant. deltaNp63α is a p63 variant that lacks the transactivation domain (TA) normally located at the N-terminus. This isotype acts as a repressor on the same target genes where the TA isoforms activate transcription. Bakkers and colleagues [119] identified deltaNp63α as a direct target of BMP signaling and as a repressor blocking neural development. They later found that both NEDD4-mediated ubiquitination and UBC9-mediated sumoylation were critical regulators of the stability of deltaNp63α protein [120]. This function turned out to be relevant for the regulation of dorsal–ventral patterning during gastrula stages. The authors found that both UBC9 and the E3-ubiquitin ligase NEDD4 showed restricted expression on the dorsal side of the embryo where deltaNp63α is destabilized. When mRNA of *deltaNp63α* mutant forms that cannot bind to UBC9 or NEDD4 is injected in embryos, the neural repressor effect of deltaNp63α extends to the entire neuroectoderm, showing the importance of ubiquitination and sumoylation on the dorso-ventral patterning of the neural region. Additional analysis in zebrafish also implicated the deltaN isoform of p63 in pectoral fin development [119], suggesting that limb malformations in humans could be related to this isoform as well.

The last example of protein stability regulation by sumoylation is different from the previously discussed examples in that SUMO conjugation does not directly stimulate ubiquitin modification, but instead regulates the nucleocytoplasmic shuttling of an E3-ubiquitin ligase (MDM2) and determines its accessibility to its target (p53), which is regulated through the proteolysis system. SENP2 is a central player in this pathway that interacts with sumoylated MDM2 and regulates its SUMO conjugation, thereby mediating its subcellular distribution. These conclusions were formed based on analysis of a *Senp2* null allele designed for the inactivation of all of the different forms of the *Senp2* gene products [89]. These mice show lethality at midgestation and are unable to form a healthy placenta as a result of deficiencies in the formation of various trophoblast cell types (Fig. 1). Therefore, these authors focused on extra-embryonic development. Analysis of the cell cycle indicated that progression was defective in all stem cell niches of the trophoblast in *Senp2* mutants because SENP2 is essential for the G1-S transition. Investigation of the downstream targets underlying this phenotype led to the identification of the p53–MDM2 pathway for regulation of trophoblast development. MDM2 is a RING finger E3-ubiquitin ligase that binds and controls the cellular levels of p53. It was already described that MDM2 is sumoylated in vivo and that this process regulates the stability and activity of MDM2 towards p53 [121–123]. In this report, Chiu et al. [89] extended these findings, demonstrating that sumoylation of MDM2 dictates its subcellular distribution. Modified MDM2 preferentially accumulates in the nucleus, where it cannot regulate p53. By contrast, desumoylated MDM2, which can move freely to the cytoplasm, is capable of p53 degradation. SUMO modification of MDM2 is regulated by

SENP2, and the loss of this enzyme causes dislocation of MDM2, leading to aberrant stimulation of p53. This study highlighted a novel connection between SUMO modification and the induction of polyploidy with the p53-MDM2 circuit as the mediator and provided additional evidence of sumoylation in cell-cycle progression.

Effects of SUMO-conjugation on epigenetic regulation

Epigenetic regulatory factors play important roles in the regulation of cell identity and fate. Polycomb group (PcG) genes were identified as being essential in these epigenetic developmental processes. These genes encode components of multimeric transcriptional repressor complexes, which act together with chromatin remodeling factors and other components to mediate global silencing of various developmental transcription factors (e.g., HOX, GATA, TBX, and SOX genes). Among the PcG proteins, PC2/CBX4, which associates with the polycomb repressive complex 1 (PRC1), was identified as a SUMO E3-ligase [124]. In addition, several mammalian PcG proteins, including PC2/CBX4 and EZH2 and SUZ12 in the polycomb repressive complex 2 (PRC2), were reported to be sumoylated [125, 126]. Therefore, sumoylation could be functionally relevant for PcG-mediated silencing. The recent characterization of another mouse SENP2 mutant allele by Kang et al. [88], uncovered some connections between sumoylation and epigenetic regulation. SENP2 mutant mice die at around embryonic day 10 (Fig. 1). Their main abnormality is related to myocardial development and is associated with a markedly reduced cellular proliferation of cardiomyocytes. Senp2$^{-/-}$ embryos also display downregulation of GATA4, GATA6, and MEF2C, which are all transcription factors that control cardiac development [88]. The expression of *Gata4* and *Gata6* is tightly regulated by PRC1 during development. Accordingly, PRC1 exhibited an increased repressive activity in these mutant embryos. In this study, the authors used an siRNA approach in mouse embryonic fibroblast cultured cells to demonstrate the role of SENP2 in the regulation of the expression of PcG target genes. Then, studies using ChIP assays indicated that SENP2 acts through alternating its occupancy of PC2/CBX4 on its promoters. They also used antibodies to show that sumoylation of PC2/CBX4 markedly enhances its binding to the histone trimethylation marker H3K27me3 in the chromatin of PcG target genes. Similar biochemical studies in transfected COS-1 cells revealed that SENP2 specifically deconjugates sumoylated PC2/CBX4. From these results, the authors concluded that ablation of SENP2 in embryos leads to excessive sumoylation of PC2/CBX4, which in turn facilitates binding of PRC1 to methylated chromatin, hence increasing the transcriptional repression of PcG-regulated genes such as *Gata4* and *Gata6*. Notably, not all of the PcG target genes were affected by the SENP2 mutation, implying that only certain PcG targets are regulated by the SENP2-PC2/CBX4 pathway. Future studies on the regulation of SENP2 throughout development and on the localization of this protein within the nucleoplasm are needed. It is currently unknown whether the E3-ligase activity of PC2 is involved in its self-sumoylation.

The PcG SOP-2 protein is involved in HOX silencing in *C. elegans*. Inactivation of SOP-2 leads to HOX ectopic expression, causing homeotic transformations [127]. Interestingly, global reduction of sumoylation phenocopies the ectopic HOX phenotypes observed by inactivation of SOP-2 [127]. Wild-type SOP-2 is sumoylated and locates in nuclear bodies. When a SUMO protease is overexpressed, SOP-2 is homogenously distributed inside and outside the nucleus, suggesting that sumoylation of SOP-2 is essential for its correct location in nuclear bodies and subsequent repression of HOX genes [127]. The effect of sumoylation on the *Drosophila* PcG protein Sex Comb on Midleg (SCM) is also interesting. This protein binds to a Polycomb Response Element (PRE) involved in the silencing of the HOX gene *Ultrabithorax* (*Ubx*) [128]. ChIP analyses showed that SCM is not recruited to the PRE at the same levels when sumoylated compared to the non-sumoylated form. Levels of *Ubx* expression respond to the sumoylation status. When the SUMO protease Ulp1 is knocked down, increasing the amount of sumoylated SCM, *Ubx* expression is higher than normal. By contrast, knocking down SUMO in the haltere, where *Ubx* is normally expressed, causes the haltere to wing transformation, which is the phenotype shown by *Ubx* silencing in the thorax [128].

Thus, sumoylation has the contrary effect in *Drosophila* SCM to that observed in *C. elegans* SOP-2. While SOP-2 sumoylation allows its silencing function and its correct localization, SCM sumoylation impedes its binding to its target sequence.

The SUMO pathway in germ cell development and meiosis

A number of studies have reported the expression patterns of genes related to the sumoylation pathway in different germ cell populations of *Drosophila*, mice and humans [129–134]. These studies support the participation of sumoylation in different steps of spermatogenesis (Fig. 1). In mice, SUMO1 and other sumoylation proteins were found in leptotene/zygotene spermatocytes, prepubertal and adult pachytene spermatocytes [131]. In humans, synaptonemal complex (SC) proteins SCP-1 and SCP2 were shown to be sumoylated by SUMO1 during pachytene [130]; moreover, SUMO1 was localized to gonosomal chromatin during zygotene when chromosome homologues pair and in XY bodies during meiotic sex chromosome inactivation [134].

This localization implicated sumoylation in meiotic chromosome dynamics. In mouse oocytes, SUMO1 is localized to the spindle poles in prometaphase I, metaphase I and metaphase II stages, around the separating homologues in anaphase I and telophase I stages of first meiosis, whereas SUMO2/3 mainly concentrates near the centromeres during mouse oocyte maturation [135]. Overexpression of in vitro produced *Senp2* mRNA in oocytes caused inhibition of SUMO-modified proteins and resulted in defects in metaphase II spindle formation in mature eggs, suggesting that the SUMO pathway might be indispensable for oocyte meiotic maturation. Evidence for the involvement of sumoylation in meiosis has also been shown in other species. In yeast, different findings linked sumoylation with the formation of the synaptonemal complex. In addition, Zip3, the master regulator of SC assembly, was identified as a SUMO E3-ligase [136] and Red1, an axial component of the SC, was found to be a sumoylation substrate [137]. Additionally, UBC9 has been shown to be associated with the SC [138, 139], an observation that was initially made in early studies with hamster spermatocytes, where UBC9 was identified as an SC component [140]. In *Drosophila*, *lwr*, the gene encoding UBC9 [129], was named because a mutant allele of this gene was originally identified as a suppressor of the female meiotic mutation caused by the *nod* (*no distributive disjunction*) *Dominant Ted Wright* allele (nod^{DTW}) that affects spindle formation. Further characterization of the original *lwr* and its derivatives showed that they are able to suppress nondisjunction defects caused by different classes of meiotic mutants, leading to the hypothesis that defective UBC9 function may affect some element of the material that holds chromosomes together during meiosis. Therefore, the correlative expression data in mammals is very significant.

Additional results showed elevated expression of SUMO and other components of the pathway during spermiogenesis. SUMO1 localizes in the chromocenters of certain round spermatids and perinuclear ring and centrosomes of elongating spermatids, implicating SUMO-1 in the process of microtubule nucleation and nuclear reshaping [131, 134]. High levels of SUMO are found in post-meiotic spermatocytes at the early canoe stage in *Drosophila*, when histones are removed and substituted by protamines [141]. Moreover, all the components required for sumoylation are co-expressed in *Drosophila* pole cells, suggesting a role of this pathway in germ-line gene expression [142]. In light of these observations and considering the above-described data regarding the reduction of PIASx (leading to a subtle phenotype in mice testis) and the remarkable participation of sumoylation in gametogenesis and vulva formation in *C. elegans* [57], a specific and important role of sumoylation in meiosis and germ cell development is expected. For this reason, designing functional experiments that reveal the essentiality of sumoylation in these processes is needed.

Concluding remarks

Sumoylation is a very effective strategy for modulating the activity of several developmental regulators. All mechanisms known to influence cell development (through sumoylation) have been evidenced in living embryos, including regulation of transcription, protein stability or shuttling of proteins in the cell. Importantly, localization of transcription factors and organization of genes into specific nuclear compartments with distinct transcriptional environments are recurrent themes.

The participation of sumoylation in developmental processes is not merely confined to fine tuning activities within signaling cascades. In some cases, the sumoylation state can be a determinant for the cell fate, as it can trigger specific differentiation programs.

Because sumoylation is transient, its regulation is critical. For adequate use of sumoylation in development, many mechanisms of specific regulation are expected to exist. E3-ligases and SENPs have emerged as central regulators of the sumoylation levels during embryogenesis. The data corroborating that both families of proteins discriminate between substrates in vivo are very relevant. Numerous questions remain in this area, including how the E3-ligases and SENPs regulate themselves and how they connect with a specific signaling pathway or sense different environmental factors. The interplay between sumoylation and other post-translational modifications, such as acetylation, phosphorylation, and ubiquitination, is another potential level of regulation for specific substrates.

The PIAS proteins are often involved during development in a SUMO conjugation-independent manner, leading to interesting questions from an evolutionary point of view. Understanding how promoters can sense the sumoylation state of a transcription factor in vivo remains an important question to be addressed.

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